RESPIRATION OF HOMOGENIZED EMBRYOS: RANA PIPIENS AND RANA PIPIENS ♀ × RANA SYLVATICA ♂[1]

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Embryos belonging to the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ cleave and blastulate normally, but the normal sequence of gastrulation movements does not occur (Moore, 1946). Such embryos remain alive, but suspended in a morphological state superficially similar to that of a very young gastrula. Before the occurrence of the developmental block, the respiration of hybrid embryos is quantitatively similar to that of normal *R. pipiens* controls, increasing exponentially with age; but for most of the period following its occurrence, their respiration is characterized by a function whose value is a constant (Barth, 1946). For an account of the attempts that have been made to analyze the biochemical and morphogenetic peculiarities of this hybrid, the reader is referred to the review by Gregg (1957).

The problem of explaining the respiratory peculiarities of gastrula-blocked hybrids is of course closely connected with that of constructing a theory to account for the exponential respiratory increase characterizing the pre-hatching development of normal embryos. In connection with this latter problem, suggested explanations have tended to fall into at least two classes: (1) those which postulate an increasingly rapid developmental synthesis of respiratory enzymes or substrates, and (2) those which assume a progressive increase in the structural availability of respiratory enzymes to their substrates. Each of these is supported by at least some of the available evidence. On the assumption that one or both of those types of explanation is well-founded, it is plausible to suggest two corresponding sorts of explanation to account for the post-blastula deficiencies of hybrid respiration: (i) that there is a failure to continue the synthesis of respiratory enzymes or substrates at a sufficient rate, or (ii) there is a failure of some developmental process which normally continues to increase effective contact of respiratory enzymes and their substrates.

In this paper we report the results of some simple homogenization experiments which it is hoped will have some bearing on these various questions.

Methods

Fertilizing, rearing and staging embryos. For fertilizing and rearing embryos, the following routine was adopted. Two separate sperm suspensions, one of *R. pipiens* sperm and one of *R. sylvatica* sperm, were prepared simultaneously in two fingerbowls, N and H. Half of the ripe eggs from a gravid *R. pipiens* were stripped into N and half into H. After two or three hours, the *R. pipiens* embryos in N were separated with scissors into small groups and distributed among several fingerbowls $N_1, \ldots N_n$; the hybrid embryos in H were similarly dispersed among several bowls $H_1, \ldots H_n$. Embryos in pairs of bowls (N_i, H_i) were reared at

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similar temperatures, ranging from 8° C. to 25° C., as convenient. Thus, the normal embryos in bowl N_1 served as controls for the hybrids in the corresponding bowl H_1. The medium in the bowls, 10% amphibian Ringer's solution without phosphate or bicarbonate, was changed daily. At the desired stages, obtained by reference to the tables of Shumway (1940), embryos were freed of their jelly coats with jeweler's forceps, re-staged, and homogenized. Hybrid embryos were assigned the stage-numbers characterizing the developmental stages of normal control embryos.

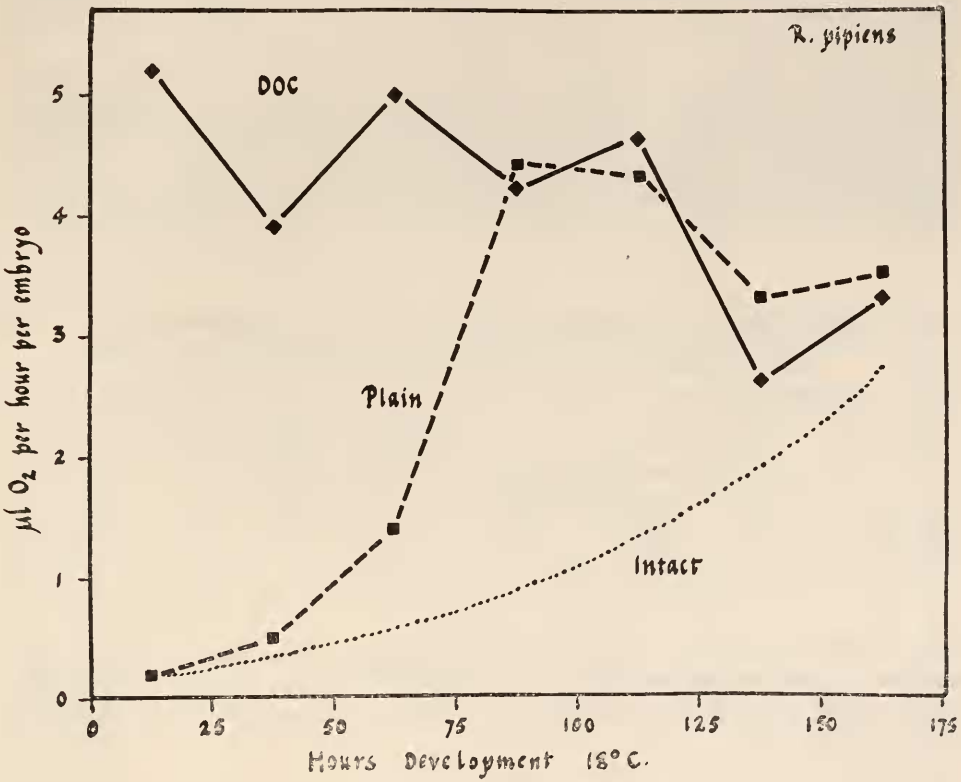

FIGURE 1. Respiration of homogenized *R. pipiens* embryos. Lower curve for intact *R. pipiens* embryos constructed from data of Moog (1944).

Preparation of homogenates. Cell-free breis were prepared by homogenizing batches of *n* jelly-free embryos in 0.05 *n*-ml. aliquots of suitable ice-cold media, using a high-speed homogenizer manufactured by the Lourdes Instrument Corporation. Two sorts of media were used routinely:

(a) 0.01 *M* phosphate buffer made up in 10% amphibian Ringer's solution without phosphate or bicarbonate, pH 6.8–7.0 Breis prepared in this medium will be called *plain-breis.*

(b) Medium (a) with the addition of 0.2% sodium deoxycholate (DOC). Breis prepared in this medium will be called *DOC-breis.* (We are indebted to Dr. W. H. Berg for suggesting the use of deoxycholate.)

Measurement of brei respiration. Within 10 minutes after preparation, 1.0-ml. aliquots of cold homogenate (about 20 embryos) were pipetted into 7-ml. Warburg flasks rigged for the measurement of oxygen uptake. After a period of temperature equilibration in the respirometer bath (about 10 minutes), manometer readings were begun and continued at 6-minute intervals for 45–60 minutes. The temperature of the respirometer bath was controlled at 24° C. The flasks were shaken continuously at a rate of 100–110 complete cycles per minute, at an amplitude of 6–8 centimeters. The rates of oxygen uptake were calculated from the readings taken during the first 30 minutes and are expressed as microliters (μl.) of oxygen per hour per embryo.

Treatment of data. In Shumway's tables, each stage s is correlated with a unique time $t(s)$, namely, the time required for a normal embryo to develop from fertilization to that stage, at 18° C. Furthermore, each $t(s)$ falls in exactly one of the successive 25-hour intervals following the moment of fertilization. We have made use of these correlations in presenting the results of measurements of the respiration of breis made from embryos with different environmental (temperature) his-

TABLE I

Effect of buffer concentration on respiration of plain- and DOC-breis, R. pipiens, stage 10. After equilibration period in respirometer, deoxycholate in appropriate buffer, pH 6.8–7.0, added from side-arm. Final brei concentration, 20 embryos per ml.

Plain-breis	Buffer concentration, molar	0.025	0.05	0.075	0.1	0.15	0.2
	μl. O_2 per hour per embryo	0.35	0.23	0.43	0.43	0.27	0.19
DOC-breis, 0.2% DOC	Buffer concentration, molar	0	0.01	0.02	0.04	0.07	0.08
	μl. O_2 per hour per embryo	7.8	8.0	7.9	6.4	6.6	6.9

tories. Thus, to construct Figure 1 and Figure 2, we have averaged for each 25-hour interval the respiratory rates of breis made from embryos whose stage s has a $t(s)$ in that interval and have plotted the resulting average against the interval's midpoint.

RESULTS

R. pipiens, plain-breis. Reference to Figure 1 will show that the average respiratory rate of plain-breis increases exponentially with the age of the embryos used in their preparation from an initial value of about 0.2 μl. O_2 per hour per embryo to a maximum value in the fourth 25-hour interval of about 4.5 μl. O_2 per hour per embryo, and thereafter tends to decline.

This result is in sharp contrast to that of Spiegelman and Steinbach (1945), who reported that the endogenous respiration of plain-breis prepared from embryos shortly after fertilization is already at a maximum. We are at a loss to explain the discrepancy, for we have been able to elevate the respiration of breis only by treatment with a detergent (see next paragraph), but not by altering the buffer concentration (Table I), nor, in preliminary experiments, by adding glycogen, glucose, hexose diphosphate, adenylic acid, magnesium, or various combinations thereof.

TABLE II

Effect of sodium deoxycholate concentration on respiration of homogenized R. pipiens embryos, stage 10–11. Breis and deoxycholate made up separately in 0.07 M phosphate buffer, pH 6.8–7.0. After equilibration period in respirometer bath, deoxycholate added from side-arm. Final brei concentration, 20 embryos per ml.

Final concentration of sodium deoxycholate, per cent	0	0.1	0.2	0.4	0.8
μl. O_2 per hour per embryo	0.12	2.8	6.6	5.6	4.4

R. pipiens, DOC-breis. The maximum respiratory rate attained by plain-breis, *i.e.,* that exhibited by embryos homogenized during the fourth 25-hour period of development, can be matched by that of deoxycholate-treated breis prepared from embryos of any lesser age (Fig. 1). Plain-breis and DOC-breis prepared from embryos older than this, however, respire at the same rate. The degree of respiratory elevation obtained earlier is a function of the concentration of DOC, 0.2% being the optimal concentration (Table II).

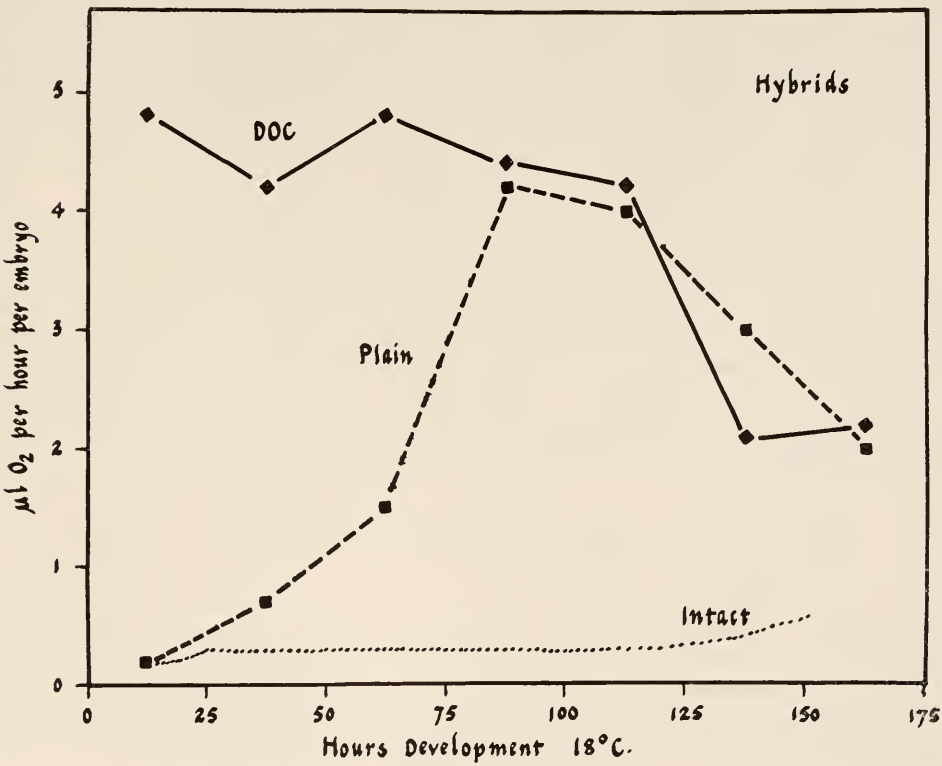

FIGURE 2. Respiration of homogenized gastrula-blocked hybrid embryos (*R. pipiens* ♀ × *R. sylvatica* ♂). Lower curve for intact embryos constructed from data of Barth (1946) and Moog (1944).

The curve for DOC-breis shown in Figure 1 is very similar to that reported for plain-breis by Spiegelman and Steinbach, although the general respiratory level is much higher and begins to decline somewhat later. We do not know if the stimulatory effect of DOC can be obtained with other detergents. However, we have found that plain-breis of early embryos respire at unusually high rates if they are prepared in Waring Blendor vessels freshly washed in Alconox. The effect wears off after several preparations without intervening cleansings with Alconox. This suggests that detergent is trapped in the bearings and leaks out slowly during the preparation of breis, but a systematic study of the question has not been made.

Hybrids, plain-breis and DOC-breis. The results of our measurements of the respiratory rates of plain- and DOC-breis made from hybrid embryos (Fig. 2) can be summarized very briefly: the respiration of such breis is quantitatively similar to that of corresponding breis made from normal control embryos, except perhaps in the seventh 25-hour period when the hybrids are moribund.

Discussion

There is no need to postulate a developmental synthesis of respiratory enzymes or substrates to account for the exponential rise of respiratory rate characterizing the development of normal amphibian embryos; for, as the high respiratory rates of DOC-breis show, there is from the outset of development enough respiratory machinery to support oxidation-rates greater than any exhibited by intact pre-hatching embryos. Similarly, there is no need to assume a synthetic failure to account for the abnormal constancy of post-blastula hybrid respiration, because the experiments with DOC-breis have shown that hybrid embryos at nearly all stages are potentially capable of as much respiration as normal controls.

For normal embryos, we adopt the conclusion of Spiegelman and Steinbach, namely, that the exponential respiratory increase is causally related to morphogenetic changes which progressively facilitate the union of respiratory enzymes and their substrates. Correspondingly, to explain the respiratory constancy of aging post-blastula hybrids, we assume that those changes somehow have been brought to a halt at the commencement of the gastrula stage. What sorts of changes might be involved is at present unknown. The elevation of embryonic respiration obtained by homogenizing in plain phosphate buffer, increasingly extensive as development proceeds, suggests that some cellular structures are becoming increasingly sensitive to mechanical disturbance or to alterations of chemical milieu, and the maximal respiration obtained at all stages by the treatment of breis with deoxycholate suggests a cellular component sensitive to detergent action: in both cases the mitochondria come immediately to mind because of their close association with oxidative enzymes of the Krebs cycle and of the hydrogen transport system, and because they are sensitive to isolative procedures, especially in the presence of deoxycholate (Siekevitz and Watson, 1956). It is reasonable to suppose that the extensive fragmentation of mitochondria by deoxycholate is only a more thoroughgoing version of what happens at any developmental stage to the mitochondria in plain-breis. In any case, it would be very interesting to study the mitochondria of differentiating embryos, and to compare those of hybrids with those of normal embryos.

The decline in the respiration obtainable from breis of late embryos may be explained by assuming, as Spiegelman and Steinbach do, a depletion of endogenous

respiratory substrates. In this connection, it is known that only half of the stored carbohydrate available at the time of fertilization remains in normal embryos at the time of hatching, although more than this remains in hybrids of the same age (Gregg, 1948).

Summary

1. The respiration of phosphate-buffered cell-free homogenates made from *R. pipiens* embryos increases exponentially with the age of the embryos up until the time at which they are in the tailbud stage, after which the rate declines.

2. Addition of 0.2% sodium deoxycholate elevates the respiration of homogenized embryos at any pre-tailbud stage to that of tailbud-breis, but has no effect upon that of breis of post-tailbud embryos.

3. The respiration of plain- or deoxycholate-treated breis is at all stages greater than or equal to that of intact embryos.

4. The respiration of breis (plain- and deoxycholate-treated) made from gastrula-arrested *R. pipiens* ♀ × *R. sylvatica* ♂ hybrid embryos is at all non-moribund stages quantitatively the same as that of control breis of normal embryos.

5. The implications of these findings are briefly discussed.

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